THIOL SYNTHETASES OF LEGUMES: IMMUNOGOLD LOCALIZATION AND DIFFERENTIAL GENE REGULATION BY PHYTOHORMONES

Clemente MR[1], Bustos-Sanmamed P[1], Loscos J[1], Navascués J[1], James EK[2], Pérez-Rontomé C[1], Becana M[1]

[1]Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, CSIC, Apartado 13034, 50080 Zaragoza, Spain.

[2]The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, UK. becana@eead.csic.es

Introduction

The thiol tripeptide GSH (γGlu-Cys-Gly) is a major antioxidant and redox buffer in plants, where it also performs critical functions in cell cycle regulation, development, sulfur transport and storage, stress response, and heavy metal detoxification. In legumes, hGSH (γGlu-Cys-βAla) may partially or completely replace GSH with presumably the same functions. The synthesis of GSH is accomplished in two sequential reactions catalyzed by γECS and GSHS, whereas the synthesis of hGSH shares the same first enzyme and then requires a specific hGSHS. A better understanding of the regulation of GSH and hGSH biosynthesis in legumes during the stress response requires a precise determination of the subcellular localization of the enzymes and a quantitative expression analysis of the genes involved. For this purpose, two types of experiments were performed. First, these proteins were immunolocalized in legumes using electron microscopy. Second, the expression pattern of the three genes was determined in the model legume *Lotus japonicus* following treatment with several hormones that are crucial for plant development and stress signaling.

Abbreviations: ABA, abscisic acid; CK, cytokinins; γECS, γ-glutamylcysteine synthetase; (h)GSH, (homo)glutathione; (h)GSHS, (homo)glutathione synthetase; IAA, indoleacetic acid; JA, jasmonic acid; PA, polyamines; SA, salicylic acid.

Immunolocalization of γECS and hGSHS in legume tissues

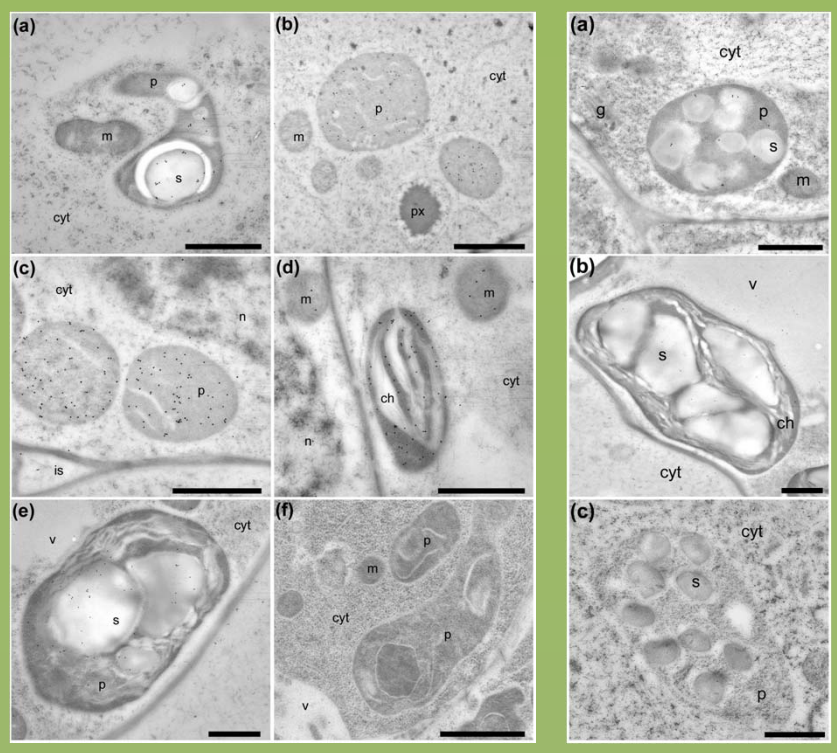

Figure 1 (left). For γECS immunolocalization, we selected two crop legumes (common bean and alfalfa) and a legume species (*Sesbania rostrata*) used as a model to study stem nodulation. We examined three plant organs with identical results. Therefore, only a summary of them is shown. The γECS protein was localized in the amyloplasts of bean root tips **(a)** and nodules **(b)**. Immunolabeling was also observed in the amyloplasts of *S. rostrata* root nodules **(c)** and in the thylakoid membranes of stem nodule chloroplasts **(d)**. In alfalfa leaves, γECS was localized to the chloroplasts, and much of the labeling was on the starch grains as well as on the thylakoid membranes **(e)**. A negative control of bean root tips in which the antibody was replaced by preimmune serum is shown **(f)**.

Figure 2 (right). The hGSHS protein was mainly localized on starch grains within amyloplasts in alfalfa roots, although there was some sparse labeling within the cytoplasm **(a)**. The pattern of localization in alfalfa leaves was somewhat similar to that in roots, with abundant immunogold labeling on starch grains in chloroplasts and less labeling within the cytoplasm and the vacuole **(b)**. A negative control of alfalfa roots in which the antibody was replaced by preimmune serum is shown **(c)**.

Abbreviations: ch, chloroplast; cyt, cytosol; g, golgi; m, mitochondrion; p, plastid; px, peroxisome; s, starch grain; v, vacuole. Bars: 1 μm.

Expression of thiol synthetases in response to hormones

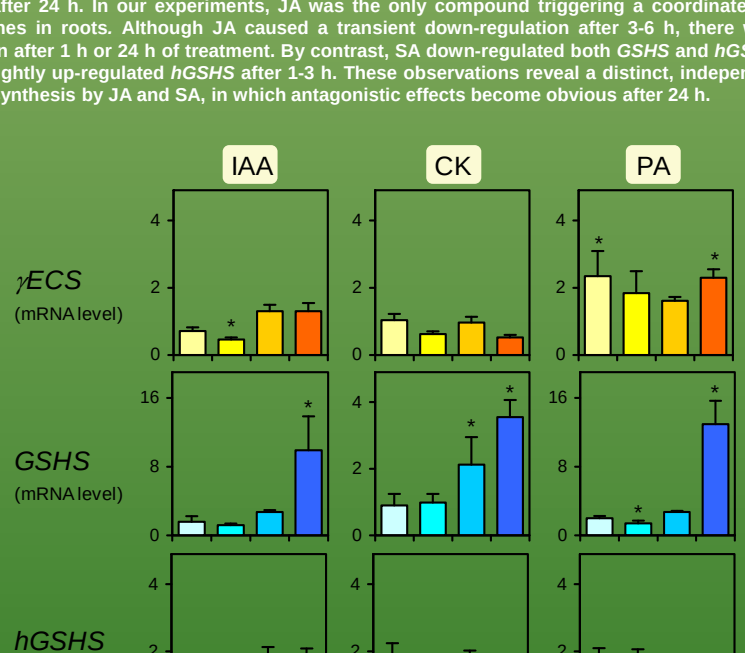

Figure 3. The regulatory mechanisms of thiol biosynthesis were studied at the transcriptional level by exposing plants to hormones. Expression of the thiol synthetase genes was examined in roots of *L. japonicus* plants grown in hydroponics. Hormones (50 μM) were provided in the rooting medium for 1-24 h. Three hormones (ABA, JA, SA) were applied to non-nodulated plants and another three (IAA, CK, PA) to nodulated plants. The mRNA levels were normalized with *ubiquitin* and expressed relative to the control values (*R*=1). Asterisks denote up-regulation (*R*>2) or down-regulation (*R*<0.5) of the genes.

(a) The application of ABA resulted in up-regulation of *γECS* after 6-24 h and in down-regulation of *GSHS* and *hGSHS* after 24 h. In our experiments, JA was the only compound triggering a coordinated response of the three genes in roots. Although JA caused a transient down-regulation after 3-6 h, there was a marked up-regulation after 1 h or 24 h of treatment. By contrast, SA down-regulated both *GSHS* and *hGSHS* genes after 24 h, and slightly up-regulated *hGSHS* after 1-3 h. These observations reveal a distinct, independent regulation of thiol biosynthesis by JA and SA, in which antagonistic effects become obvious after 24 h.

(b) Both IAA and CK up-regulated *GSHS* but had no effect on the expression of the two other genes. Induction of *GSHS* by IAA and CK would thus promote GSH synthesis, explaining some effects of both hormones on cell division and differentiation, processes that specifically require GSH. Exogenous PA slightly up-regulated *γECS*, had no effect on *hGSHS* expression, and strongly activated the *GSHS* gene in the roots after 24 h. Interestingly, the three hormones playing a role in cell division (IAA, CK, PA) induced *GSHS* but not *hGSHS*, further supporting a specific function of GSH in this process.

Expression of thiol synthetase genes, hGSHS activity, and hGSH content in roots in response to hormones

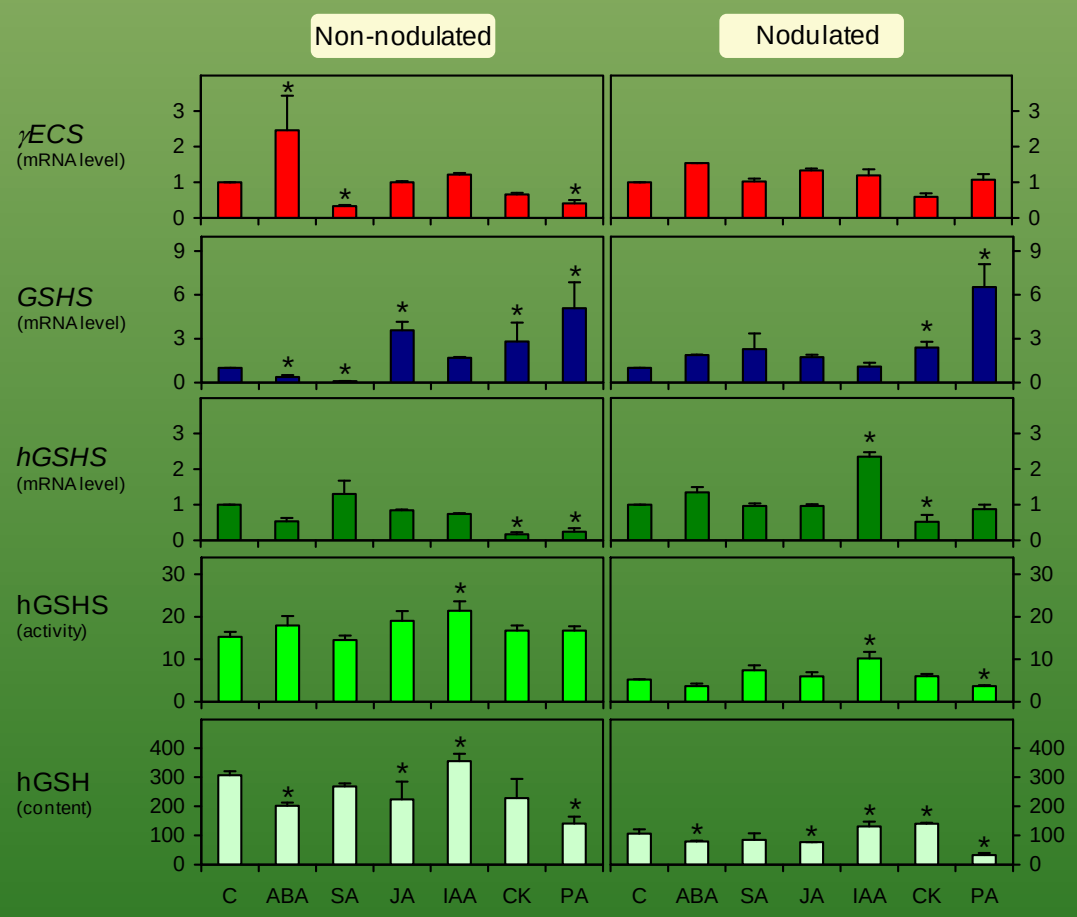

Figure 4. The effects of hormones on the mRNA levels of the three genes and on hGSHS activity and hGSH content were analyzed. The GSHS activity and GSH content were not measured as they were very low because *L. japonicus* is a hGSH-producing legume. The major results are as follows: ***(i)*** ABA increased the *γECS* mRNA level, whereas SA down-regulated *γECS* and *GSHS* in non-nodulated plants; ***(ii)*** JA increased the expression of *GSHS* in non-nodulated plants, and decreased the hGSH content in non-nodulated and nodulated plants; ***(iii)*** IAA increased hGSHS activity and hGSH content in both types of plants; and ***(iv)*** PA induced the *GSHS* gene but down-regulated *hGSHS* and decreased hGSHS activity and hGSH content in non-nodulated and/or nodulated plants. Therefore, there is a complex and differential regulation of the *GSHS* and *hGSHS* genes in response to hormones, with some effects being dependent on the nodulation status of the plants.

Units: mRNA levels (normalized to *ubiquitin* and expressed relative to control values, which were given *R*=1); hGSHS activity (nmol min^{-1} g^{-1} fw); hGSH content (nmol g^{-1} fw). For mRNA levels, asterisks denote up-regulation (*R*>2) or down-regulation (*R*<0.5). For hGSHS activities and hGSH contents, asterisks denote significant differences (*P*<0.05) with respect to the controls.

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